Renewable and Sustainable Energy Reviews 26 (2013) 332–343

Contents lists available at SciVerse ScienceDirect

Renewable and Sustainable Energy Reviews

journal homepage: www.elsevier.com/locate/rser

Status of molecular breeding for improving *Jatropha curcas* and biodiesel

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ARTICLE INFO

Article history:
Received 4 February 2013
Received in revised form
17 May 2013
Accepted 20 May 2013
Available online 22 June 2013

Keywords:
Jatropha
Biofuel
DNA marker
Genome sequencing
GWAS
GS

ABSTRACT

Jatropha curcas is believed to be one of the potential biofuel crops, as it does not compete with planting lands for the edible oil plants. However, *J. curcas* has not been domesticated for producing biodiesel. Conventional breeding to increase the productivity of *J. curcas* has started since the early 2000s. Although some genetic improvement of oil yield has been made through conventional breeding, oil yield is currently still too low (≤2000 kg/ha/year) to make the biodiesel production from *J. curcas* sustainable. Due to the enormous potential of marker-assisted selection (MAS) and genomic selection (GS) to speed up genetic gain through early selection, genomic resources such as DNA markers, a linkage map, transcriptome sequences and a draft genome, have been developed and some are being used in genetic improvement for sustainable production of biodiesel. In this review, we present the recent advances in conventional breeding, as well as development and applications of genomic resources to improve the quantity and quality of biodiesel extracted from seeds of *J. curcas*. We also highlighted the requirement of a well-assembled reference genome of *J. curcas* and the potentials of next generation sequencing (NGS) for genome-wide association studies (GWAS) and GS to speed up the increase of the yield and quality of biodiesel from *J. curcas*.

Contents

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http://dx.doi.org/10.1016/j.rser.2013.05.055

1. Introduction

Fossil fuel reserves are limited, while demand is ever-increasing worldwide [1]. Combustible fuels are the world's main energy resource and are at the center of global energy demands [2]. People are increasingly concerned about climate change [3], the dwindling supply of fossil fuel, as well as its unstable and rising costs, which has motivated researchers to seek alternative, renewable energy sources [3–5]. Biofuels are one of the solutions to energy security, the reduction of emissions of greenhouse gas and sustainable development [6]. Biodiesel has received considerable worldwide attention in the past years as it is environmental friendly [7]. However, many countries (e.g. China and Japan) do not allow the use of edible oils (e.g. soybean, palm and rapeseed oils) to produce biodiesel to ensure food security [8]. Therefore, alternative plant sources for non-edible oil for use in production of biodiesel have been extensively sought after [9].

The plant *Jatropha curcas* Linnaeus originated from Mexico and is an underutilized oil-bearing crop [10]. It was brought to Asia and Africa by Portuguese traders 350 years ago [10]. Its seeds can be processed into biodiesel and it is believed that *J. curcas* can grow on poor soils and areas of low rainfall (from 250 mm a year), hence, it has been promoted as the ideal plant for small farmers in countries such as India [11], China [12] Indonesia [13] and Africa [14]. However, *J. curcas* had never been domesticated for producing biodiesel before recent years [15]. Since 2008, several countries have started breeding programs to improve seed yield [16–19]. According to published reports, each mature tree produces an average of 4 kg of seeds per year when cultivated under optimal conditions [12,13,15]. Its oil yield is still much lower in comparison to other oil producing plant species, such as oil palm, which is the main bottleneck in plantation of *J. curcas* for production of biodiesel [20]. Besides seed yield, other traits such as the number of female flowers, later maturity, resistance to lodging, resistance to pest and disease, reduced plant height and high natural ramification of branches are also important for improving oil yield [21–23]. However, the genetic improvement for oil production with traditional breeding is very slow and tedious as phenotypes can only be measured after they are expressed.

Molecular breeding, also called marker-assisted selection (MAS), refers to the procedure of the use of DNA markers which are tightly linked to traits to assist phenotypic selection [24]. In comparison to traditional breeding, molecular breeding possesses several advantages such as selection at seedling stage, no influence of environment, and selection of preferred homozygotes, thus accelerating the genetic improvement. With the rapid development of next-generation sequencing (NGS) technologies, it is now easy to detect and characterize a large number of DNA markers using NGS and polymerase chain reaction (PCR) [24]. Molecular breeding has already been applied in important agronomic species to speed up genetic improvement, such as in rice, maize and corn [24]. In jatropha, molecular breeding is still in its infancy [25], although some reports on DNA markers [26,27], linkage map [28] and QTL mapping for seed yield [25,28,29] have been published.

Several important issues on jatropha biodiesel concerning plantation, tissue culture, biotechnological and biochemical engineering, biodiesel production and applications, economy and policy have already been reviewed [11–13,15,30]. However, this review is different from these excellent reviews, and combines relevant information about the recent advances of the development of genomic resources and their applications in accelerating genetic improvement of *J. curcas* for enhancing quantity and quality of biodiesel extracted from seeds of *J. curcas*. We also discussed the potentials of genome-wide association studies (GWAS) and genomic selection (GS) for speeding up the increase of the yield and quality of biodiesel from *J. curcas*.

2. Conventional breeding for increasing oil yield

2.1. Plantation and phenotypic variations

Jatropha curcas L. belonging to the Euphorbiaceae family is a perennial crop. Its seeds contain up to 35% oil [10]. *J. curcas* is traditionally used as a hedge plant and various parts of the tree have been collected for medicinal uses. However, *J. curcas* was not domesticated and extensively selected for oil yields before the 2000s [31]. As a result, *J. curcas* currently is still a wild plant with low oil yields. The oil yield of wild *J. curcas* is less than 1000 kg/ha/year [12,13], much lower than some major oil crops such as oil palm, coconut oil and rapeseed [32]. We have summarized the annual oil yield of major oil-producing plants in Fig. 1. Due to the realization of its potential for producing biodiesel to decrease the oil crisis, reduce pressure on the environment and control urban air pollution; and many claims about its advantages, *J. curcas* is believed to be an ideal plant for producing biofuel [33,34]. Thus, the plantation of *J. curcas* moved from small scale to large scale in India, China, Malaysia, Indonesia, Philippines, Burma, Saudi Arabia, Ghana, South Africa, Senegal, Nigeria, Tanzania, Ethiopia, Zaxmbia and Zimbabwe and other countries. In 2008, the total plantation area was 900,000 ha globally, among which 84.4% (760,000 ha) was in Asia, 13.3% (120,000 ha) in Africa and 2.23% (20,000 ha) in Latin America. According to a recent report of FAO [35], the total plantation area of *J. curcas* is expected to be 12.8 million ha by 2015. The largest producing country will be Indonesia in Asia, Ghana and Madagascar in Africa and Brazil in Latin America [35].

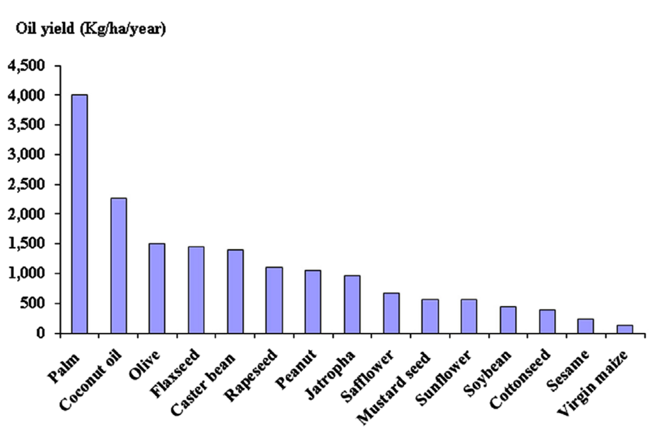


Fig. 1. Annual oil yield of major oil-producing plants. Data were extracted from several sources [32,44,45].

Substantial phenotypic variations in seed yield, tree height, branch number, flowering time, and female to male flower ratio were observed [9,11,12]. Seed yield varied from 300 to 7500 kg/ha/year [9,11,12]. However, after a few years of large scale plantation, people realized that *J. curcas* was not a wonder plant for sustainable biodiesel production on marginal land due to its very low oil yield (1500 kg/ha/year for improved varieties, see Figs. 1 and 2), and concluded that plantation of *J. curcas* on marginal land could only get marginal yield [20,36]. Even on normal fertile soils. *J. curcas* [11,22,37] was no match for other major oil producing crops [32] (see Fig. 1). On one hand, these data may reflect the difficulties in making the plantation of *J. curcas* profitable and sustainable, and on the other hand, highlight the potential for genetic improvement through breeding.

2.2. Conventional breeding and its achievements

Conventional breeding for genetic improvement of *J. curcas* has been started since the early 2000s in India, China, Thailand, Philippines, Mexico, Guatemala, and Brazil [9,11,12]. Although several traits, such as seed yield, oil contents, female to male flower ratio, synchronistic of flowering and fruiting, branch number and oil quality are important for the genetic improvement for producing biodiesel, increasing the oil yield is the priority [21,35]. In the past few years, many researches focused on collection of germplasm and selective breeding. Montes et al. undertook evaluation trials in *J. curcas* involving 225 lines from Asia, Africa and Latin America to study degree of variability [38]. Their study revealed low genetic variability in African and Indian accessions and high genetic variability in Guatemala and Latin American lines. Evaluation of *J. curcas* for phenotypic and genotypic variations by Basha and Sujatha [39] showed similar results, indicating a lower genetic variation in *J. curcas*. Selective breeding is the core activity of genetic improvement for oil production [40]. Mass selection, recurrent selection, hybrid breeding and induced mutation breeding were applied to improve trait performances. Details about these methods for genetic improvement can be found in the review by Divakaran et al. [21].

In India, mass breeding started in the early 2000s. The National Oilseeds and Vegetable Oils Development Board, India collected over 5000 accessions with a network of 40 institutions and identified 1855 candidate plus trees. Department of Biotechnology, India, collected 1500 accessions [41]. Kaushik et al. analyzed oil content and kernel seed coat ratio for 1000 samples of seeds from 12 states in India, and found that the collection from Uttaranchal had the highest percentage (73%) of high oil yielding plants [42]. Most of the *J. curcas* varieties were developed from selections made in the natural populations [10,43]. The first variety, SDAUJ I (Chatrapati), was identified as the best among 496 seed sources [44] for commercial cultivation in the semi-arid and arid regions of Gujarat and Rajasthan in India. According to a recent review by Pandey [31], the dry seed yield of *J. curcas* in India is still too low (<6 t/ha) to be profitable, and earlier claims of high seed yield could not be proved by serious studies.

In China, around 100 accessions were selected for further examinations after examination of over 800 [12]. The annual yields of 10 varieties of *J. curcas* were higher than 2.5 kg/tree and kernel oil content was $>65\%$. After 4-years of plantation trial from six different sources, Yang et al. selected one with higher yield. The oil yield of the selected variety was 1566 kg/ha, which is more than five times the national best yield [45]. Over 100 copies of elite germplasm were marked using inter-simple sequence repeat (ISSR) [12,46]. More than 500 copies of materials were mutated with chemical mutagenesis, Co60 radiation and space carrying technologies [12]. Ten good seed sources, one new variety and 10 mutants were selected and identified in the field based on the biological characteristics, economic traits, resistant characteristics and other indicators [12]. In China, variety breeding for *J. curcas* is lagging behind its large scale plantation [12]. The lack of high yield varieties is one of the main hurdles for developing the industry of *J. curcas* in China [45,47].

In Africa and South America, mass breeding has been conducted in several countries in co-operation with some European countries. According to a recent presentation of Dr Van Loo (personal communication) and other reports [35], the best oil yield was 1500 kg/ha/year. In addition to improving the oil yield, Zimbabwe has developed non-toxic varieties of *J. curcas*, which would make the seed cake following oil extraction suitable as animal feed without its detoxification (http://precedings.nature.com/documents/2658/version/1). However, its oil yield is not yet known.

The Singapore Company JOil (S) Pte in co-operation with the Temasek Life Sciences Laboratory has started breeding *J. curcas* since 2006. Field tests showed that the seed yield of their selected varieties already reached at 2.4 t/ha on poor land in India in the first year. Small scale tests on selected elite varieties showed that, under good conditions, the oil yield could reach 2000 kg/ha/year (http://www.joil.com.sg/Latest-News-Archive). The British company, BP-D1, reported that the oil yield of several elite varieties were as high as 2000 kg/ha/year under good management [48]. We have summarized the expected and the realized highest oil yield of *J. curcas* reported in scientific journals and conferences in Fig. 2. Based on scientific reports, we estimated that the maximum oil yield of *J. curcas* could be 4 t/ha/year.

Although a number of institutes are involved in breeding, it seems that the results of breeding have not been published. On the other hand, a number of commercial companies (e.g. BP-D1) have already released their breeding results on their websites, which seem to be very promising. However, these data need to be further proved by large-scale field examination by other parties. Although the conventional breeding of *J. curcas* for genetic improvement has already increased the yield of oil, the improvement is very slow. About 5–7 years are required to obtain improved cultivars through conventional breeding. Furthermore, oil quality is very difficult to be improved through conventional breeding approaches. MAS is expected to accelerate the genetic improvement not only for seed yield, but also for traits which are difficult to select for, such as

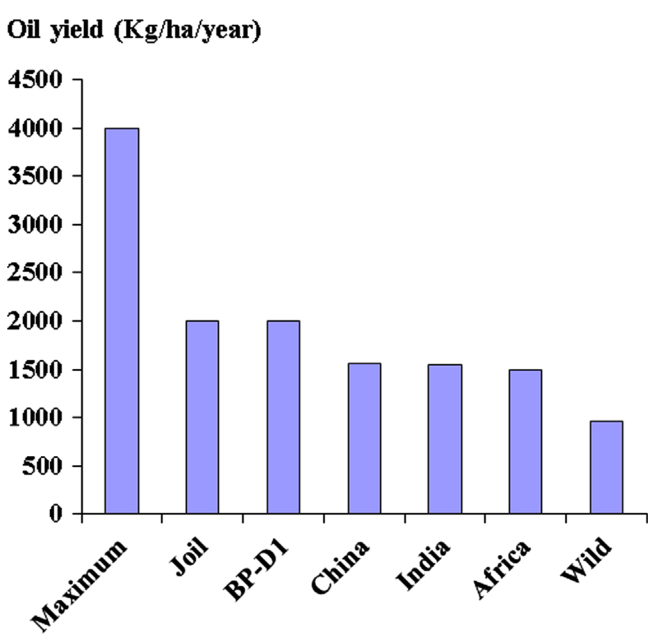


Fig. 2. Annual oil yield of improved varieties of jatropha. Data were extracted from several sources [10,31,35,44,45,48].

oil quality and disease resistance [49]. For MAS, DNA markers closely linked to important traits must be identified. DNA markers must be cloned, characterized and mapped to the whole genome in order to identify these associations [50,51].

3. Genomic resources for speeding up the increase of oil yield and quality

Genomic resources such as molecular markers, linkage maps, ESTs and genome sequences are powerful tools to speed up genetic improvement for oil yield and quality through MAS or GS [52,53]. The major institutions working on molecular breeding of *J. curcas* can be found in Table 1. Availability of selected genomic resources in *J. curcas* is summarized below.

3.1. DNA markers

In a genome, most of the DNA sequences are conserved among individuals, while a small proportion is variable. DNA markers are the variable DNA sequences in a genome that can be differentiated using molecular or biochemical methods. Among all genomic resources, DNA markers have direct use for germplasm characterization, linkage and QTL mapping and molecular breeding [54]. Currently, although several old types of DNA markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and inter-simple sequence repeat (ISSR) markers have been used in studying genetic variations in natural and cultured populations of *J. curcas* [55–57], two types of DNA markers (i.e. microsatellites [58] and single nucleotide polymorphism (SNP) [59]) are the most preferred in linkage mapping and studies on genetic diversity for agronomic species. This is mainly because microsatellites and SNPs are highly abundant and easy for cost-effective and high throughput scoring. More recently, a new type of DNA polymorphism, copy number variation (CNV), has been reported in humans and model organisms [60]. The application of CNV in agronomic species just came into sight recently [61]. However, in *J. curcas*, no CNV has been reported.

RAPD, ISSR and AFLP have been used in analyzing genetic variations of wild and cultured varieties of *J. curcas* and their relationships [16,39,46,55,56,62–68]. The general finding is that the genetic diversity is very low in *J. curcas*.

Microsatellites have already been identified and used in *J. curcas*. In 2008, our lab presented over 300 microsatellites at an international conference on *J. curcas* in Singapore [28]. Other laboratories have also identified a few microsatellites [27,69]. Microsatellites were used increasingly in *J. curcas* [28,56,69–72] in the evaluation of germplasma and genome mapping. Currently, due to the advent of NGS technologies [73], resequencing of a genome of 3 giga bases costs less than 1000 USD using the Illumina's Hiseq 2000. Using bioinformatic software (see review [74]), microsatellites can be easily detected in genome sequences.

Table 1
Major institutions involved in molecular breeding of *Jatropha curcas* worldwide.

Country	Institution	Major activities	References
Singapore	Temasek Life Sciences Lab	Microsatellites/SSR, SNP, genes, linkage and QTL mapping, sequencing transcriptome and genome, MAS, transgenic jatropha, tissue culture	[25,28,29,82,87,102,110,111]
	Joil Pte	AFLP, sequencing genome, MAS, transgenic jatropha, tissue culture	[112]
India	Biotech Park	DNA markers, ESTs, genes,	[65,112]
	Osmania University	AFLP	[65]
	Central Salt & Marine Chemical Research Institute	RAPD, AFLP, SSR	[70,71,113]
	Tamil Nadu Agricultural University	RAPD, ISSR, ESTs, genes,	[114–116]
	SRM University	ESTs, gene,	[117,118]
	Dhirubhai Ambani Life Sciences Center	ESTs	[119]
China	Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences	ESTs, genes, ISSR, RAPD	[16,79]
	South China Agricultural University	ESTs, genes, SSRs, AFLP	[16,79,120]
	South China Botanical Garden, Chinese Academy of Sciences	SSR, RAPD, AFLP, genes, transgenic plants	[56,101,121]
	Sichuan University	ESTs, genes, ISSR,	[122–124]
	Institute of Tropical Biosciences and Biotechnology, Chinese Academy of Tropical Agricultural Sciences	BAC library, ESTs,SSR	[26,125]
Japan	Kazusa DNA Research Institute	Genome sequencing	[89]
Thailand	Annamalai University	Mutagenesis, microsatellites, ISSR	[126,127]
		RAPD	[127]
	Kasetsart University	ISSR, microsatellites, genes	[126,128,129]
Philippines	University of the Philippines Los Baños	Genetic variation	[130]
Brazil	Universidade Estadual de Santa Cruz (UESC),	ESTs, genes,	[131]
	Universidade Catolica de Brasília–SGAN	RAPD	[132]
	State University of Campinas, UNICAMP	ESTs, genes	[133]
Indonesia	Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development	RAPD	[134]
Malaysia	University Putra Malaysia	ISSR	[135]
The [136]	Netherlands	Plant Research International B.V.	Molecular genetics
USA	SG Biofuels	SSRs, SNP, genome sequencing	[137]
UK	BP-D1	DNA markers	[48]
Africa	Biotechnology Laboratory, Kenya Forestry Research Institute	RAPD	[62]

The draft genome sequence of *J. curcas* [75] is already available; microsatellites can be easily identified, which could save a substantial amount of money and time for development of DNA markers.

SNPs have recently been identified. By sequencing pooled samples, Silva-Junior et al. identified a total of 18,225 SNPs in 11.9 giga bases, suggesting extremely low frequency of SNPs in *J. curcas* [76]. Recently, Gupta discovered 2482 informative SNPs by sequencing 148 global collections of *J. curcas* lines and found that a narrow level of genetic diversity existed among the indigenous genotypes as compared to the exotic genotypes of *J. curcas* [77]. Our group developed some SNPs in ESTs and used them in constructing a linkage map of jatropha [28]. Although some SNP markers are now available *J. curcas* and could be very useful in molecular breeding for substantial improvement of biodiesel yield and quality, no cost-effective and high throughput genotyping platforms were developed for *J. curcas*.

3.2. A linkage map

A linkage map is the essential framework for genome-wide identification of associations between DNA markers and traits [51]. One or several segregating populations where DNA markers segregate are required to construct a linkage map. Population sizes varied from dozens to a few hundred individuals. For high-resolution mapping, a large number of individuals (>200 individuals) are required. In *J. curcas*, due to the very low DNA variation, it is difficult to construct a highly informative reference family for linkage mapping [28]. Therefore, families generated by interspecies crosses are the better choice. According to previous work on inter-species hybridization, *J. curcas* can hybridize with species *Jatropha integerrima*, *Jatropha canascens,* and *Jatropha gossypifolia* [21].

A first-generation linkage map was constructed using a mapping population containing two families consisting of 96 individuals. The families were produced by interspecies (*J. curcas* x *J. integerrima*) cross and backcross [28]. The mapping population was genotyped with co-dominant DNA markers (i.e. SSRs and SNPs in genes). A total of 506 markers (216 microsatellites and 290 SNPs from ESTs) were mapped onto 11 linkage groups. The length of the map was 1440.9 cM, with an average marker spacing of 2.8 cM. Blasting the 222 ESTs containing SSR and SNP markers mapped on the linkage map against EST-databases revealed that 91.0%, 86.5% and 79.2% of *J. curcas* ESTs were homologous to counterparts in castor bean, poplar and *Arabidopsis* respectively. 192 orthologous markers of *J. curcas* were mapped to the assembled whole genome sequence of *Arabidopsis thaliana*. 38 syntenic blocks were detected with the comparative mapping. Small linkage blocks were well conserved, but often shuffled. The linkage map and the data of comparative mapping laid the foundation for QTL mapping of agronomic traits, MAS and cloning genes responsible for phenotypic variations. An additional 500 microsatellites and SNPs have already been genotyped and will be mapped to the existing linkage map of *J. curcas*. Although other researcher groups claimed that they were constructing linkage maps for *J. curcas*, so far no other linkage maps have been reported. The slow progress of linkage mapping in *J. curcas* may be due to the low genetic variations in its natural resources, which makes the generating of informative reference families for mapping difficult. Therefore, we recommend using informative families generated by crossing different species (e.g. *J. curcas, J. integerrima, J. canascens* and *J. gossypifolia*) for linkage mapping.

3.3. Transcriptome

The transcriptome is the complete set of all RNA molecules, which include mRNA, tRNA, rRNA, miRNA and other non-coding long or short RNA in one or a population of cells. The transcriptome can vary in different cells, tissues and with external environmental conditions. Studying the dynamics and regulation of the transcriptome is critically important in the understanding of the functions of a genome and the underlying biological processes. Several projects were initiated to sequence expressed sequence tags (ESTs) of *J. curcas*. In Genbank, there are over 100,000 EST sequences deposited. Costa et al. sequenced 13,249 ESTs from developing and germinating seeds [78]. They identified most known genes related to lipid synthesis and degradation. They also detected ESTs coding for proteins that may be involved in the toxicity of seeds. In addition, they found a high number of ESTs (800) containing transposable element-related sequences in the developing seed library when contrasted with those found in the germinating seed library. Chen et al. established three cDNA libraries with mRNA from embryos at different developmental stages [79]. They sequenced ESTs and obtained 9844 unique sequences of which 1070 were contigs and 3595 were singletons. Yadav et al. constructed a normalized and full-length enriched cDNA library from developing seeds [69]. The library contained about 1×10^6 clones with an average insert size of 2.1 kb. They sequenced a total of 12,084 ESTs using Sanger sequencing. The average length of the high quality reads was 576 bp. After assembly, 2258 contigs and 4751 singletons were obtained. Annotation of these 7009 unisequences by BLASTX revealed that most (6386/7009) of the unisequences could be annotated. 6233 of the 7009 unisequences were identified to be potential full-length genes. Functional classification revealed these unisequences covered a broad range of cellular, molecular and biological functions. King et al. recently conducted high-throughput sequencing analysis of the transcriptome of developing seeds using 454 sequencing [80]. Using a single sequencing run, they obtained 46 Mb of raw sequence data including 95,692 sequences. After assembly, they yielded 12,419 contigs and 17,333 singletons. They found that storage proteins were the most abundant transcripts. They observed that metallothioneins, ribosomal proteins, and late embryogenesis abundant proteins were also highly expressed. Curcin, which is a type-I ribosome-inactivating protein, was also abundant accounting for 0.7% of the transcriptome. Purushothaman and Madasamy conducted 454 pyrosequencing of normalized cDNAs from flowers, mature leaves, roots, developing seeds and embryos of *J. curcas* [81]. They obtained 381,957 high-quality reads from 383,918 raw reads. After assembly, they got 17,457 contigs and 54,002 singletons. The assembled transcripts averaged 916 bp in length. 2589 of these transcripts were full-length. The authors discovered that 2320 transcripts were related to major biochemical pathways including the oil biosynthesis pathway. By comparisons with other publically available ESTs of jatropha, 14,327 assembled transcripts were novel. Silva et al. [76] sequenced ESTs from polled RNA using two lanes of the Illumina sequencing platform [76]. They obtained 11.8 giga bases of high-quality sequence. Gu et al. constructed several cDNA libraries for different tissues [82] and sequenced over 50,000 EST clones using Sanger sequencing. A number of genes related to the synthesis of fatty acids were obtained. However, the complete data set has not been published.

The large number of transcripts will serve as an invaluable genetic resource for genetic improvement of *J. curcas*, and sequence information of genes involved in the biosynthesis of fatty acids could be used for metabolic engineering of *J. curcas* to increase oil content, and to modify oil composition. However, a complete reference transcriptome of *J. curcas* is still unavailable. Transcriptomics studies require a high quality, comprehensive reference transcriptome that includes all transcripts, coding and noncoding, large and small RNA [83], therefore, it is essential to conduct an assembly of all available EST data using sophisticated software (e.g. CLC Genomics workbench) to get a well assembled and annotated comprehensive transcriptome of *J. curcas*.

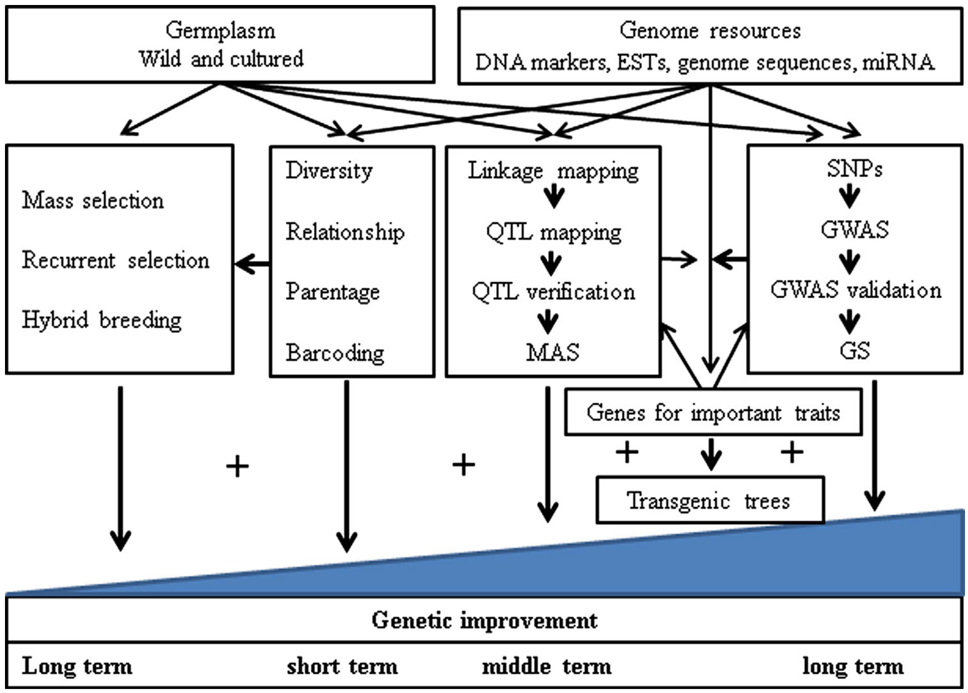


Fig. 3. Application areas of genomic resources in improving *J. curcas*. In short term (1–2 years), genomic resources can be used in analyzing genetic diversity, population relationships and parentage, as well as barcoding elite trees. In middle term (2–4) years, genomic resources can be applied to linkage and QTL mapping and marker-assisted selection (MAS) and producing transgenic trees for improving yield and quality of biodiesel. In long term (> 4 years), genomic resources can be utilized to identify a large number of SNPs on the whole genome, to conduct whole genome association studies (GWAS) and genomic selection (GS) for accelerating genetic improvement of *J. curcas*.

3.4. miRNAs

miRNAs, which are small noncoding RNAs, play crucial regulatory roles in gene silencing by targeting mRNAs g [84]. Due to of the ability of miRNAs to inactivate either specific genes or entire gene families, artificial miRNAs can function as dominant suppressors of gene activity when they are brought into a plant. Consequently, miRNA-based manipulations of gene functions have emerged as promising new approaches for genetic improvement of crops. This includes the genetic modification of agronomic traits and the development of new breeding strategies [85]. This strategy has been used in genetic improvement of fruits [85,86]. In *J. curcas*,52 putative miRNAs were identified by sequencing 2000 clones from a small RNA library of leaves and seeds [87]. Among them, six were identical to known miRNAs and 46 were novel. Quantitative real-time PCR revealed differential expression patterns of 15 miRNAs in root, stem, leaf, fruit and seed. Ten miRNAs were highly expressed in fruits and seeds, suggesting that they are involved in seed development or fatty acids synthesis in seeds. In addition, 28 targets of the isolated miRNAs were predicted by using 20,000 EST sequences from a cDNA library [82]. These miRNA target genes encode a broad range of proteins. Sixteen targets were associated with genes belonging to the three major gene ontology categories of biological process, cellular component, and molecular function. Four targets were identified for the miRNA JcumiR004. By silencing JcumiR004 primary miRNA, expressions of the four target genes were up-regulated and oil composition was modulated significantly, indicating diverse functions of JcumiR004. Vishwakarma et al. recently identified 22 miRNAs from ESTs and genome survey sequences [88]. However, the number of miRNAs identified in *J. curcas* is still limited. Further identification of additional novel miRNAs is essential. It can be expected that the applications of miRNAs in genetic improvement in *J. curcas* will emerge soon.

3.5. Genome sequence

The genome of *J. curcas* has been sequenced by several research institutes (e.g. Synthetic Genomics USA; ACGT, Malaysia; Temasek Life Sciences Laboratory, Singapore; Kazusa DNA Research Institute, Japan) and companies (e.g. Life Technologies and SG Biofuels, USA). However, only the results generated by Japanese scientists have been published [75,89]. By integrating *de novo* assembly of a total of 537 million paired-end reads generated from the Illumina sequencing platform into the previous genome assembly [75], a new assembly was reported recently [89]. The newly assembled genome was 297.7 Mb consisting of 39,277 contigs. The average and N50 lengths of the generated contigs were 7579 and 15,950 bp, respectively [75,89]. In addition, the authors collected all available transcriptome data from the public databases and assembled them into 19,454 tentative consensus sequences. By comparing these tentative consensus sequences of transcripts, and updating genome sequences, the authors predicted a total of 30,203 complete and partial structures of protein-encoding genes. The number of genes with complete structures was substantially increased in comparison to the previous genome annotation. The authors further analyzed the number and features of the tandemly arrayed genes, syntenic relations between *J. curcas* and other plant genomes, and structural features of transposable elements. The detailed information on the updated *J. curcas* genome is available at http://www.kazusa.or.jp/jatropha/. It is expected that the draft genomic sequence and accompanying information will serve as valuable resources for speeding up fundamental and applied research of *J. curcas*. However, it is of note that the assembled genome is still a draft genome sequence. Further efforts on filling gaps, linking scaffolds to linkage groups/chromosomes, and identifying DNA markers by resequencing additional individuals are essential.

Genomic tools by themselves will not increase the productivity and sustainability of the production of oil from *J. curcas*. However,

they will provide more approaches to accelerate genetic improvement for increasing jatropha oil yield and quality. What is needed now is to use the genomic tools to facilitate every step of the breeding of *J. curcas* to increase the yield and quality of oil.

4. Applications of genomic resources in improving biodiesel production

Genomic resources summarized above have been used or are being applied in accelerating both basic and applied research for genetic improvement of jatropha for biodiesel production. Application areas of genomic resources in *J. curcas* are summarized in Fig. 3. Some selected areas demonstrating applications of genomic resources are given below.

4.1. Accessing genetic variations

Genetic variations in natural populations are the sources of genetic improvement. Therefore, information about genetic variations is critically important in any breeding program. Genetic variations in natural and cultured populations around the world have been studied by using RAPD, ISSR, and AFLP [16,39,46,55,56,62–69]. The general finding is that the genetic variations in *J. curcas* in the varieties from Asia, Africa and Brazil are lower whereas the genetic variations are slightly higher in varieties from Mexico [16,63,90,91], thus supplying a scientific base for selective and hybrid breeding. The studies on genetic diversity using microsatellites obtained very similar results as those obtained using RAPD, ISSR and AFLP assays. Our lab has studied genetic diversity of 278 individuals of *J. curcas* collected from four continents using an automated DNA sequencer ABI 3730 × l (Applied Biosystems). Surprisingly, we found that there was no genetic variation (see example of marker genotyping data in Fig. 4) at all the 29 microsatellite loci (Unpublished data). In addition, our lab has constructed the first linkage map of jatropha using microsatellites and SNPs developed by us (see details below). 216 microsatellites and 290 SNPs mapped in the linkage map [28], were all homozygote in the *J curcas* mother, but all heterozygous in the *J. integerrima* x *curcas* hybrid father. Recent SNP analysis showed that the genetic variations in *J. curcas* accessions were very low [77]. All these data suggest that the variations at DNA level are extremely low in *J. curcas*.

4.2. Molecular barcoding of elite trees

DNA profile analysis can be used to distinctly identify individual animals. Once the unique DNA profile is obtained, the information can be used to differentiate individuals [92]. In comparison to physical tagging, the genetic identity using DNA profiling is more reliable, and cannot be changed and modified by humans. In tree breeding, an important issue is to protect the results of the lengthy breeding programs, namely the selected elites. Once elite trees are selected, it can be easily multiplied by tissue culture. Physical tags

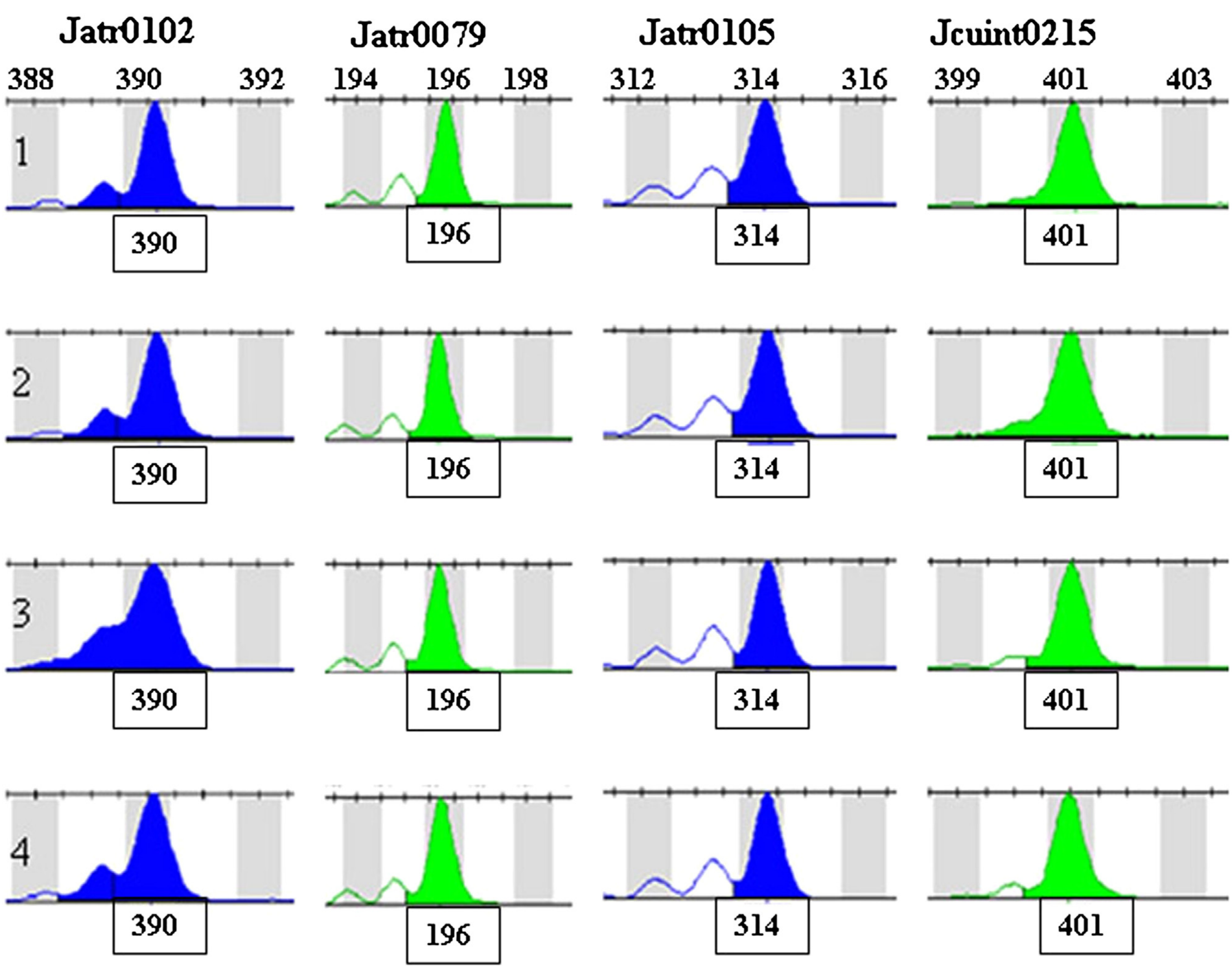


Fig. 4. Genotypes at for four SSR loci (Jatr0102, Jatr0079, Jatr0105, and Jcuint0215) showing that all individuals (1–4) of *J. curcas* are homozygous and share the same genotype at each locus.

can certainly be used to label elite trees. However, it can be easily lost and changed. The ideal way is to tag the elites using their own DNA profiling. Usually, the possibility of two individuals sharing the same genotypes was 10^{-8} when using 8–10 microsatellite markers [93,94]. In *J. curcas*, a similar genetic barcoding system using 11 microsatellites located on 11 linkage groups [28] has been developed by our group, and is being used in identifying and protecting elite trees (Unpublished data). However, due to low microsatellite variation in pure *J. curcas*, the identification power could be also low. In the hybrid varieties generated by crossing *J. curcas* with *J. integerrima*, and backcrossing, the power of identification of the 11 markers could be higher. However, to save time and cost, it is essential to develop a multiplex PCR to amplify all markers in one PCR [94].

4.3. Identifying candidate genes for important traits

Besides the identification of DNA markers [69], genomic resources (e.g. ESTs) can be used for transcript profiling to identify the candidate genes for traits of interest, as well as development of microarray to study differential expressions of different genes in different tissues at different developing stages.

Genes related to fatty acids synthesis, stress resistance and other important traits have been isolated from cDNA libraries and RNA-seq using NGS. For example, Tan et al. isolated the JcERF gene, which is an ERF subfamily member. They found that the full-length JcERF functioned effectively as a trans-activator in the yeast one-hybrid assay [95]. In transgenic *Arabidopsis*, overexpression of JcERF enhanced the salt and freezing tolerance, whereas the seed germination was not affected. Their results suggest that JcERF functioned as a novel transcription factor. To identify novel genes expressed during stress in *J. curcas*, Eswaran et al. conducted a screen of a cDNA library constructed from salt-stressed roots and obtained 32 full-length genes that can confer abiotic stress tolerance [96]. These genes could be over-expressed to generate and evaluate transgenic plants for stress tolerance as well as be used as markers for breeding salt stress tolerance. Jang et al. obtained the JcDof1 gene from seeding, and confirmed that this gene was located in the onion epidermal cell nucleus [97]. This gene exhibited DNA-binding and transcriptional activation activities in yeast. The JcDof1 expression was characterized by a circadian-clock oscillation under long day, short day and continuous light conditions, suggesting that JcDof1 was a circadian clock-Dof transcription factor gene responding to light signals. A putative flowering-time-related Dof transcription factor gene, JcDof3 was cloned and characterized by Jang et al. Recently, Gu et al. cloned and characterized genes accA, accB1, accC and accD that encode the subunits of heteromeric ACCase [98]. They found that the accA, accB1, accC and accD genes were temporally and spatially expressed in the leaves and endosperm. More recently, 68 fatty acids and lipid biosynthetic genes have been identified from a normalized cDNA library constructed using cDNA from developing endosperm. Gu et al. investigated their expression at different developing stages of endosperm [82]. They found that the expression of the majority of fatty acid and lipid biosynthetic genes was highly consistent with the development of oil bodies and endosperm in seeds, while the genes encoding enzymes with similar function may be differentially expressed during endosperm development.

In addition to the isolation of candidate genes related to important traits, a method for rapid analysis of *J. curcas* gene functions by virus-induced gene silencing has been developed recently [99]. The method produced robust and reliable gene silencing in plants agroinoculated with recombinant TRV harboring jatropha gene sequences. The virus induced gene silencing (VIGS) method can be used for high-throughput screening of jatropha genes and analysis of their functions.

4.4. QTL mapping for oil yield and quality

Most economically important traits such as plant height, seed yield, and oil content in seeds are quantitative in nature, and are controlled by many genes, environmental factors and their interactions. In most cases, the underlying single genes have small effects. Quantitative trait loci (QTL) are gene clusters or chromosomal regions influencing the expression of a quantitative trait [100]. QTL mapping can facilitate the understanding of the number and effects of genes that determine the expression of a trait and assist in selective breeding to accelerate genetic improvement [24,51]. In genetic improvement of jatropha seed yield, oil content, oil composition, tree height, branch number, disease resistance and pest resistance are the important traits. QTL mapping has been conducted for some of these traits.

Using 105 microsatellites almost evenly covering 11 linkage groups (LGs) of the linkage map of jatropha, and a backcrossing population with 296 jatropha trees, a total of 28 QTL for tree growth and seed traits were mapped on the whole genome [25]. Two QTLs *qTSW*-5 and *qTSW*-7 for seed yield were located on LGs 5 and 7 respectively. In these two LGs, two QTL clusters harboring five and four QTL respectively controlling yield related traits were detected. These two QTL clusters played pleiotropic roles in regulating seed yield and plant growth. Positive additive effects of the two QTL indicated higher values for the traits conferred by the alleles from *J. curcas*, while negative additive effects of the five QTL on LG 6, controlling plant height, branch number, female flower number and fruit number respectively, demonstrated higher values conferred by the alleles from *J. integerrima*. Therefore favored alleles from both the parents could be integrated into an elite jatropha plant by further backcrossing and MAS.

The major fatty acids in seed oil of jatropha are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). High oleic acid and total oil content are desirable for jatropha breeding. Composite interval mapping detected 18 QTL for oil traits on the genome of jatropha [29]. A highly significant QTL *qC18:1*-1 was detected at one end of LG 1 explaining 36.0% of the phenotypic variance (PVE). The QTL *qC18:1*-1 overlapped with *qC18:2*-1, influencing the contents of oleic acid and linoleic acids. Among the significant QTL controlling total oil content, *qOilC*-4 was mapped on LG 4 with PVE of 11.1%. Meanwhile, oleosins are the major composition in oil body affecting oil traits. Three oleosin genes *OleI*, *OleII* and *OleIII*, were mapped onto the linkage map of jatropha using SNPs in these genes. *OleI* and *OleIII* were mapped on LG 5, close to QTLs controlling oleic acid and stearic acid. QTL (eQTL) for the expressions of the three genes were mapped on LGs 5, 6 and 8 respectively. The eQTL for *OleIII*, *qOleIII*-5, was located on LG 5 and overlapped with QTL controlling stearic acid and oleic acid, implying a cis- or trans-element for the *OleIII* affecting fatty acid compositions.

While QTL for some important traits have been mapped, many important traits such as disease resistance and pest resistance, which are critically important for the sustainable development of the jatropha industry for biodiesel production, have not been studied yet. To make MAS possible, fine mapping and confirmation of mapped QTL are essential.

4.5. Marker-assisted selection and introgression

Introgression of recessive genes and pyramiding of multiple genes are very difficult using conventional breeding methods [53]. However, MAS is useful to overcome such problems. Several genes can be pyramided either for the same trait or for different traits along with faster recurrent parent genome recovery through intense background selection. In addition, MAS can be used to introgress a lot of recessive genes in less time than conventional

breeding [53]. In the case of *J. curcas*, some studies have already been initiated to use the molecular markers in breeding programs. Liu et al. identified QTL for high oleic acid and total oil content, and recommended integrating the QTL for selection of elite trees [29]. More recently, Sun et al. recommended using pleiotropic QTLs regulating plant growth and seed yield [29]. However, some time is still needed before the outcome of MAS can be seen.

Apart from the introgression of genes/QTLs linked to traits from the elite cultivars in the variety of interest, molecular markers are helpful for introgression of genes from wild species, which are generally inferior in agronomic performance, into elite cultivars. In a previous QTL analysis, a QTL linked for high branch number were detected in *J. integerrima* [29]. The authors suggested that using markers linked to these QTL can transfer the useful genes from *J. integerrima* to *J. curcas*, while stringent background selection is necessary to limit linkage drag by tracking the presence of unwanted genomic segments of *J. integerrima*. However, the results of these efforts have not been reported, and are expected to be known soon.

5. Bioengineering for improving jatropha for the production of biodiesel

Genome and transcriptome sequences have been used in identifying genes which play an important role in fatty acid synthesis, and their promoter regions. The information of the coding and regulatory regions is critically important in modifying fatty acid synthesis pathways using transgenic technologies to improve the quality and quantity of biodiesel production from *J. curcas*. Transformation approaches for generating transgenic jatropha have already been optimized [101–103]. Researches on modifying fatty acid synthesis pathways through transgenic technologies have been initiated in several countries, such as Singapore, China, Japan and India leading to improve the quality and quantity of oil from *J. curcas*. However, only a few data have been published.

The ignition quality, heat of combustion and oxidative stability of oil is affected by its fatty acids profile. An ideal biodiesel contains high percentage of monounsaturated fatty acids and less polyunsaturated acids. Oil form *J. curcas* contains 30–50% of polyunsaturated fatty acids (mainly linoleic acid), which negatively impacts the oxidative stability and causes high rate of nitrogen oxides emission. In Singapore, three types of the enzyme 1-acyl-2-oleoyl-sn-glycero-3-phosphocholine delta 12-desaturase (FAD2), which are the key enzymes responsible for the production of linoleic acid in plants were identified through a whole genome approach. Using the RNA interference technology [102], the FAD2-1 was down-regulated in a seed-specific manner. The transgenic JcFAD2-1 plants increased oleic acid ($>78\%$) and there was a corresponding reduction in polyunsaturated fatty acids ($<3\%$) in its seed oil, thus enhancing the quality of its oil. The presence of high seed oleic acid did not have a negative impact on other jatropha agronomic traits (e.g. oil yield). This is probably the world's first genetically modified jatropha plant for increasing the quality of biodiesel from *J. curcas*. Field trials and commercialization of the transgenic trees started in 2013.

Yin et al. from the Temasek Life Sciences Laboratory, Singapore filed a patent (US 2012/0073018 A1) on the isolation of *J. curcas* curcin genes, tissue-specific promoters and the production of curcin-deficient jatropha plants. By using RNAi transgenic technology, curcin gene expression was suppressed, thus substantially reducing the amount of curcin protein in seeds and leaves that is harmful to human health. These transgenic plants reduced toxic effect of *J. curcas* on people working on *J. curcas*. However, it is not known whether the suppression of the expressions of the curcin genes will influence the performances of other economic traits (e.g. seed yield, oil quality, resistance to pests and diseases).

In Japan, in an attempt to improve drought tolerance for sustainable production of biodiesel from *J. curcas*, three kinds of transgenic jatropha plants were generated [104]. The first one is the transgenic plant in which the *PPAT* gene, which encodes an enzyme that catalyzes the CoA biosynthetic pathway, was over-expressed. The second over-expressed the *NF-YB* gene, which encodes a subunit of the NF-Y transcription factor; whereas the third the *GSMT* and *DMT* genes, which encode enzymes that catalyze production of glycine betaine, were up-regulated. Preliminary results suggest that the expressions of the introduced *GSMT* and *DMT* genes significantly enhance glycine betaine synthesis in jatropha, and thus should effectively improve the drought tolerance of jatropha.

In China, transgenic plants with ω-3 fatty acid desaturase FAD8, that catalyzes the dienoic acid rapidly to produce trienoic acid in cold conditions, have been generated for improving the cold resistance in seedlings of *J. curcas* [105]. Some biological parameters related to cold tolerance in the transgenic plants suggest that the transgenic trees are tolerant to cold. However, no field test data have been released.

Although the experimental data of transgenic jatropha for increasing oil quality, cold and draught resistance and reducing toxicity are very promising, so far, it seems that the transgenic jatropha trees have not been gone through extensive field tests. The general productivity of these transgenic plants for producing oil under commercial plantation conditions is unknown. Besides the improvement of the targeted traits, oil yield must be maintained or improved in the transgenic plants.

6. Future directions in increasing biodiesel yield and quality from *J. curcas*

The current public and private interest in jatropha has triggered large-scale investments and expansion of its plantations. Genetic improvement for increasing oil yield using conventional breeding approaches has been initiated in some countries. However, the current oil yield is still too low to make the jatropha plantation profitable and sustainable. Genomic resources have been developed for speeding up the genetic improvement of *J. curcas* and some have already been used in the evaluation of the genetic diversity in natural and cultured population, in constructing linkage map and in mapping QTL for some important traits. However, jatropha genomics has lagged far behind that of model and other agricultural systems. It is essential to develop a high density linkage map to find DNA markers associated with high oil yield. With availability of the draft genome sequences and transcriptome, this task should not be difficult. Although QTL analyses have been carried out for identifying DNA markers associated with oil yield and quality in populations generated by interspecies hybridization, most QTL were only mapped in large marker space. Only these QTL with moderate-to large effects were detected with the current experimental design. Further confirmation and fine mapping of identified QTL for oil yield and quality in different populations are essential for future MAS. No QTL for oil yield and quality in the pure breed *J. curcas* has been reported, probably because genetic variations in *J. curcas* are too narrow.

Because the QTL mapping only identifies QTL with moderate to large effects, QTL with small effects are missed. Due to the rapid development of high-throughput and cost-effective genotyping of a large number of DNA markers (e.g. SNP), researchers are starting association mapping based on linkage disequilibrium (LD) using a large number of DNA markers covering the whole genome in model organisms, humans, livestock and agronomic plant species. The approach to associate many DNA variations (e.g. $>500{,}000$ SNPs)

in the whole genome with traits from many individuals (e.g. > 1000), is called genome wide association studies (GWAS) [106,107]. This technique has discovered the associations of particular genes with a number of common diseases in humans [106,107]. Because GWAS are based on LD, they are able to detect very small effects of marker-trait associations. For GWAS, usually natural populations are used. If alleles at markers are significantly associated with superior phenotypes, these markers can be used for selection across breeding populations. The marker-assisted selection using DNA markers associated with traits of interest which are identified in GWAS, is called genomic selection (GS). A previous study showed that breeding values can be predicted with high accuracy using SNPs along the whole genomes [108]. In *J. curcas*, GWAS could be even more attractive, as the genome of *J. curcas* is very small (ca. 400 Mb). For GWAS, the cost for genotyping could be much lower in *J. curcas* than species with bigger genomes. In addition, GS in jatropha would have other advantages: large training populations can be easily obtained. The extent of LD could be very high in superior trees with a small effective population size frequently used in current breeding programs. The recent development of genotyping by sequencing (e.g. RAD-seq) [109] has drastically reduced the cost of genotyping SNPs, which makes GWAS and GS feasible. However, GWAS requires a well-assembled references genome. Therefore, research priority should be put on acquiring a well-assembled and annotated reference genome sequence. This could be accomplished in the near future, as the Japanese scientists have already assembled a draft genome of *J. curcas*. It is expected that in the next few years, the cost of genotyping by using NGS will be reduced substantially (at least 10 folds). In the near future, GWAS promises to yield numerous SNP markers that could be used in GS for early selection of superior alleles associated with a wide range of traits (certainly also oil yield and quality) in *J. curcas*. As the efficiency of DNA sequencing, SNP discovery, genotyping and other molecular procedures improve and experimental costs decrease, the opportunities to incorporate NGS and GS into breeding programs for improving jatropha and biodiesel will substantially increase.

Another application of NGS is in studies on expressions of all genes, for which NGS, in combination of sophisticated bioinformatic tools, will surely replace microarray experiments soon. In comparison to other gene expression approaches (e.g. microarray and real-time PCR), NGS technologies can provide more comprehensive insights into the spatial and temporal control of gene expressions. Therefore, it can be anticipated that NGS will facilitate GS. NGS can also speed up the development of transgenic technologies for improving *J. curcas* and biodiesel because it becomes easier to modify genes and their regulatory elements with the increasing availability of genomic resources. Although, analysis of large sets of NGS data is still a very difficult task presently, significant progress is being made in improving existing bioinformatic and statistical tools, and in developing new algorisms and approaches for this task. We strongly believe an exponential increase in the use of NGS technologies for speeding up the improvement of *J. curcas* and biodiesel. The results of these efforts will have a profound impact on the industry of *J. curcas*.

7. Conclusion

Although *J. curcas* is a promising candidate for producing biodiesel, the genetic improvement of *J. curcas* for producing biodiesel through conventional breeding is too slow to make the production of biodiesel from *J. curcas* sustainable. Genomic resources hold the great promise in accelerating genetic improvement for sustainable production of biodiesel. Although some genomic resources have been developed and applied for genetic improvement of *J. curcas*, jatropha genomics lagged far behind that of model and other agricultural systems. Further development and application of genomic resources (e.g. a well assembled genome sequence and a large number of SNPs) are essential for rapid increasing oil yield and quality from *J. curcas*. NGS technologies will speed up the development of genomic resources, and accordingly will accelerate the genetic improvement of *J. curcas* for sustainable production of biodiesel. The future of *J. curcas* as a plant species for producing biofuel is bright.

Acknowledgments

We would like to thank our colleagues Ms. May Lee and Grace Lin for critical comments on the first version of this manuscript.

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